

Preoperative treatment with doxycycline reduces aortic wall expression and activation of matrix metalloproteinases in patients with abdominal aortic aneurysms

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Purpose: Matrix metalloproteinases (MMPs) are considered to play a central role in the pathogenesis of abdominal aortic aneurysms (AAAs). Doxycycline (Dox) has direct MMP-inhibiting properties *in vitro*, and it effectively suppresses the development of elastase-induced AAAs in rodents. The purpose of this study was to determine if treatment with Dox suppresses MMPs within human aneurysm tissue and to elucidate the molecular mechanisms underlying this effect.

Methods: Aneurysm tissues were obtained from 15 patients with an AAA, eight of whom had been treated with Dox before surgery (100 mg orally twice a day for 7 days). Protein extracts were examined by means of gelatin zymography and immunoblot analysis, and RNA was examined by means of reverse transcription-polymerase chain reaction (RT-PCR). The effects of Dox on MMP production were further examined in human THP-1 mononuclear phagocytes *in vitro*.

Results: No detectable difference was found between groups by using substrate zymography as a means of assessing total MMP activity, but Dox treatment was associated with a slight (24.4%) reduction in the activated fraction of 72-kDa gelatinase (MMP-2; $P < .05$). In contrast, a 2.5-fold reduction in the amount of extractable 92-kDa gelatinase (MMP-9) protein in Dox-treated patients was revealed by means of immunoblot analysis ($P < .05$). Also, a 5.5-fold (81.9%) reduction in MMP-9 messenger RNA (mRNA) in Dox-treated patients was demonstrated by means of quantitative competitive RT-PCR (mean \pm SE, mol MMP-9/mol β -actin: 1.3 ± 0.5 vs 7.2 ± 3.1 ; $P < .04$). There was no significant difference between groups in the relative expression of MMP-2 protein or mRNA. In cultured THP-1 monocytes stimulated with phorbol ester, the expression of MMP-9 protein and mRNA were both decreased after exposure to relevant concentrations of Dox *in vitro*.

Conclusion: In addition to its recognized effects as a direct MMP antagonist, Dox may influence connective tissue degradation within human aneurysm tissue by reducing monocyte/macrophage expression of MMP-9 mRNA and by suppressing the post-translational processing (activation) of proMMP-2. Through this complementary combination of mechanisms, treatment with Dox may be a particularly effective strategy for achieving MMP inhibition in patients with an AAA. (*J Vasc Surg* 2000;31:325-42.)

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Abdominal aortic aneurysms (AAAs) develop through a complex degenerative process associated with aging, atherosclerosis, and chronic inflammation.¹⁻⁴ Through numerous studies conducted within the last decade, a pathophysiologic concept has emerged that proteolytic degradation of medial elastin is responsible for weakening and dilatation of the aortic wall and that collagen degradation is responsible for aneurysm rupture.⁵⁻⁸ An abundant amount of evidence suggests that these alterations are mediated by members of the matrix metalloproteinase (MMP) family,^{3,9,10} including collagenase-1 (MMP-1), stromelysin-1 (MMP-3), the 72-kDa and 92-kDa gelatinases (MMP-2 and MMP-9, respectively), and macrophage elastase (MMP-12).¹¹⁻²¹ MMP-9 has attracted particular interest because it has activity against insoluble elastin fibers²²⁻²⁴ and because it is the most abundant elastolytic proteinase produced by human AAA tissues *in vitro*.¹⁶ MMP-9 is also abundantly expressed *in situ* by aneurysm-infiltrating macrophages located at the site of tissue damage,^{16,18} is synthesized in AAA tissue in a manner correlating with increased aneurysm diameter,²⁵ and is elevated in the circulation of patients with an AAA.²⁶ By using an elastase-induced model of AAA, we recently found that targeted gene deletion of MMP-9 is sufficient to prevent experimental aneurysmal degeneration in mice (Pyo et al, manuscript submitted). These observations have fostered the idea that MMP-9 and other metalloproteinases might provide useful biological markers of aortic aneurysm disease and potential targets for pharmacologic therapy in patients with a small asymptomatic AAA.¹⁻⁴

Consistent with the notion that inflammatory cell production of elastolytic MMPs plays an important role in aneurysmal degeneration, the development of experimental AAAs has been shown to be suppressed by leukocyte-depleting antibodies,²⁷ glucocorticoids,²⁸ nonsteroidal anti-inflammatory agents,^{29,30} and direct MMP antagonists.³¹⁻³³ Doxycycline and other tetracycline derivatives also inhibit the development of elastase-induced aneurysmal degeneration in the rat, in which they exhibit a greater therapeutic efficacy than any other agent tested to date.³⁴⁻³⁶ Studies examining the effect of tetracyclines on experimental AAAs were based on the recognition that these compounds display pronounced MMP-inhibiting properties, as first elucidated by Golub and colleagues in experimental models of periodontal disease.^{37,38} Like naturally occurring tissue inhibitors of metalloproteinases (TIMPs), tetracyclines are known to exert direct inhibition of MMP activities, as

demonstrated by *in vitro* assays. Non-antibiotic chemically modified tetracyclines have a similar efficacy as MMP inhibitors, demonstrating that the MMP-inhibiting properties of tetracyclines are unrelated to their antimicrobial effects.³⁸⁻⁴² Because tetracyclines are safe and effective at the dose schedules typically used in clinical practice,⁴³⁻⁴⁶ they have been successfully tested in several conditions associated with elevated MMP activity and connective tissue destruction (eg, rheumatoid arthritis, osteoarthritis, and periodontal disease).^{44,47,48} Although this indicates that tetracyclines might have similar use as metalloproteinase inhibitors in patients with an AAA, it is not yet known if these agents can effectively suppress MMPs in the complex tissue environment of degenerative human aortic aneurysms.

It has recently become apparent that direct inhibition of metalloproteinase activity, as measured by means of *in vitro* assays, may be only one of several mechanisms by which tetracycline derivatives prevent MMP-mediated matrix degradation.⁴² For example, it has been revealed by means of studies in cell culture that tetracyclines can also suppress the production of at least some MMPs, through mechanisms involving selective downregulation of gene transcription.⁴⁹⁻⁵² Furthermore, the normal extracellular processing associated with the activation of proMMPs may be altered in the presence of doxycycline, resulting in diminished activation, accelerated enzyme degradation, and loss of enzymatic activity.⁵³ These findings suggest a broad spectrum of mechanisms by which tetracyclines might inhibit MMP-mediated matrix degradation, including direct inhibition of enzyme activity, suppression of extracellular proenzyme activation, and downregulation of messenger RNA (mRNA) synthesis. Which of these mechanisms might be most important during clinical use of tetracyclines remains unknown.

With a view toward the possibility that tetracycline derivatives might be used to suppress the degradation of aortic wall structural proteins in patients with an AAA, we sought to determine whether clinically applicable treatment with doxycycline has an influence on the production of MMPs in human aneurysm tissue. The results of this investigation demonstrate that even short-term treatment with doxycycline is associated with a significant reduction in the expression of MMP-9 protein and mRNA, both in human aneurysm tissues *in vivo* and in a human monocytic cell line in culture. We also found that treatment with doxycycline promotes a reduction in the post-translational processing (activation) of proMMP-2 in the diseased aortic wall.

These findings indicate that, through a combination of favorable molecular mechanisms, treatment with doxycycline may be a particularly effective strategy for achieving MMP inhibition in patients with an AAA.

MATERIALS AND METHODS

Patients, doxycycline treatment, and tissue specimens. Eight patients scheduled for elective repair of an infrarenal AAA were treated with doxycycline. After providing informed written consent according to a protocol approved by the Washington University School of Medicine Human Research Subjects Committee, each patient was provided with a supply of 100 mg doxycycline hyclate capsules (Danbury Pharmacal, Danbury, Conn). Doxycycline was administered twice daily for at least 7 days before surgery, including the morning of the planned procedure. At the time of aneurysm repair, a full-thickness specimen of aortic wall was obtained from the point of maximal dilatation. Control AAA tissues were obtained in a similar manner from seven untreated patients undergoing elective aortic aneurysm repair during the same period. Each aortic tissue specimen was snap-frozen in liquid nitrogen and stored at -80°C before protein and nucleic acid extraction.

Substrate zymography. Protein extracts were obtained from frozen aortic tissue samples as described,²¹ by pulverizing under liquid nitrogen and extracting in ice-cold 50 mmol/L Tris-HCl buffer, with a pH of 7.5, containing 1.0 mol/L NaCl, 2.0 mol/L urea, 0.1% (w:v) Brij-35, 0.1% ethylenediamine tetraacetate, and a mixture of serine, cysteine, and aspartic protease inhibitors (Protease Inhibitor Cocktail No. P8340; Sigma Chemical, St. Louis, Mo). After centrifugation at 10,000g (1 hour at 4°C), the supernatant was centrifugally concentrated by using a 5,000 molecular weight cut-off membrane (2 hours at 4°C). Samples (10 μg total protein per lane) were subjected to gelatin zymography under non-denaturing conditions, as described.¹⁶ After staining gels with 0.5% Coomassie Blue R-250 in 40% methanol/10% acetic acid (Sigma Chemical), gelatin-degrading activities were observed as clear bands against a dark background of intact substrate. The relative molecular weight of each proteolytic band was determined by comparison with the migration of authentic human MMP-9 and MMP-2 and molecular weight standards (Bio-Rad, Hercules, Calif). Gels were dried, scanned, and analyzed by means of inverse densitometry. The relative proportion of each enzyme present in the active form was calculated from densi-

tometric measurements as a fraction of the total amount of activity present for that enzyme.

Immunoblot analysis. Aortic extract samples were normalized to total protein (100 μg per lane) and resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, by using 12% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), blocked with 5% powdered milk in 20 mmol/L Tris HCl, pH 7.5, 0.5 mol/L NaCl, 0.1% Tween-20, and incubated overnight at 4°C with monoclonal antibodies to MMP-9 or MMP-2 (Research Diagnostics, Flanders, NJ). Peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) was used as the secondary antibody, and immune complexes were visualized by means of enhanced chemiluminescence (ECL) with kit reagents (Amersham Life Science, Arlington Heights, Ill). The relative density of immunoreactive MMP-2 or MMP-9 was determined for each sample, and the amount of each MMP recovered in tissue extracts from the doxycycline-treated and untreated groups was recorded as the mean plus or minus SEM.

Reverse transcription-polymerase chain reaction and Southern blot analysis. Aortic tissue samples were pulverized under liquid nitrogen, and total RNA was isolated by means of guanidium iso-thiocyanate-phenol-chloroform extraction with Trizol reagent (Gibco BRL, Grand Island, NY), as described.²¹ The integrity of the RNA from each sample was verified by means of agarose gel electrophoresis. All samples were normalized to the same amount of total RNA, and each sample was analyzed in duplicate, along with control samples for genomic DNA (absence of reverse transcriptase) and nonspecific DNA contamination (absence of RNA template). Reactions were performed on a Gene AMP 2400 PCR thermal cycler system from Perkin-Elmer (Norwalk, Conn). First strand complementary DNA (cDNA) synthesis was performed in a total reaction volume of 20 μL using 0.5 μg of total RNA, 20 units RNase inhibitor, 2.5 $\mu\text{mol/L}$ random hexamers, 1 mmol/L dNTPs, and 50 units murine Moloney leukemia virus reverse transcriptase, as provided in the GeneAmp RNA polymerase chain reaction (PCR) kit from Perkin-Elmer/Roche Molecular Systems (Branchburg, NJ). Samples were incubated at 42°C for 15 minutes before terminating the reaction by heating to 99°C for 5 minutes, then cooling to 5°C . Reverse transcription products served as the template for PCR amplification, using primer pairs specific for human MMP-2, MMP-9, and β -actin

Table I. Oligonucleotides used in reverse transcription-polymerase chain reaction and Southern blot analysis

Human MMP-2, GenBank accession no. J03210 (anticipated product, 385 bp)		
Forward primer:	5'-GAGTACTGCAAGTTCCCTT-3'	(bp 644-663)
Reverse complement:	5'-TGTTGCCAGGAAAGTGAAG-3'	(bp 1009-1028)
Complementary DNA probe:	5'-GGCACATCCTATGACAGCTG-3'	(bp 848-867)
Human MMP-9, GenBank accession no. J05070 (anticipated product, 401 bp)		
Forward primer:	5'-AATCTCACCGACAGGCAGCT-3'	(bp 131-150)
Reverse complement:	5'-CCAACTGGATGACGATGTC-3'	(bp 512-531)
Complementary DNA probe:	5'-GGCAGATTCCAAACCCCTTGA-3'	(bp 332-351)
Human β -actin, GenBank accession no. X00351 (anticipated product, 195 bp)		
Forward primer:	5'-ATCGTGCGTGACATTAAGG-3'	(bp 632-650)
Reverse complement:	5'-ACAGGACTCCATGCCAGG-3'	(bp 808-826)
Complementary DNA probe:	5'-CTGGACTTCGAGCAGGAGAT-3'	(bp 671-690)

bp, Base pair.

(Table I). PCR amplifications were performed in a 100 μ L reaction volume with 10 mmol/L Tris-HCl buffer containing 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 100 pmol (each) forward and reverse complement primers, and 2.5 units of AmpliTaq DNA polymerase. Reactions included 4 minutes at 95°C for denaturation and 30 cycles of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C; samples were then incubated for 7 minutes at 72°C for final extension before holding at 4°C. A 30 μ L aliquot of each sample was resolved by 1.5% agarose electrophoresis in the presence of 5 ng/mL ethidium bromide, and DNA was visualized under ultraviolet light to detect the presence of PCR amplification products at the anticipated size (Table I).

Reverse transcription-polymerase chain reaction (RT-PCR) products were transferred by means of standard Southern techniques to Hybond N+ nylon membranes (Amersham Life Science). Hybridization was performed with cDNA oligonucleotide probes specific for MMP-9, MMP-2, or β -actin (Table I), by using an ECL 3'-oligolabeling and detection system (Amersham). After prehybridization, membranes were incubated at 42°C for 90 minutes with 10 ng/mL labeled cDNA. Membranes were washed under stringent conditions, incubated with horseradish peroxidase-conjugated anti-fluorescein antibodies followed by ECL detection reagents, and exposed to radiographic film. For semiquantitative analysis, the relative density of each MMP band was compared with that obtained for β -actin from the same sample, and each experiment was conducted at least twice for each RNA sample. The mean plus or minus SEM of relative density ratios was determined for all samples from each experimental group.

Competitive reverse transcription-polymerase chain reaction analysis. When sufficient RNA was available, competitive RT-PCR was also used for more accurate quantification of MMP-9 mRNA lev-

els in aortic tissue specimens.⁵⁴ The MMP-9 competitor (347 base pair [bp]) was constructed from full-length human MMP-9 cDNA by means of a modification of methods described by Celi et al,⁵⁵ then subcloned into pGEM-T Easy (Promega, Madison, Wis) and verified by means of direct sequencing (ABI PRISM Model 377; Perkin-Elmer/Roche Molecular Systems). When amplified in parallel with the same primer pairs used for MMP-9, the competitor cDNA produced a product that differed in size by 104 bp from the native cDNA product. A competitor constructed for human β -actin produced a PCR product 80 bp shorter than that obtained from the native β -actin cDNA.

After RT, competitive PCR reactions for MMP-9 and β -actin were performed in total reaction volumes of 20 μ L containing 50 mmol/L KCl, 4 mmol/L $MgCl_2$, 0.4 mmol/L dNTP, 2.5 units of AmpliTaq DNA polymerase, and 100 pmol (each) forward and reverse primers. Each reaction contained a known quantity of the competitor cDNA in a series of at least four different concentrations for each specimen examined. PCR products were separated on 1.5% agarose containing ethidium bromide and photographed under ultraviolet light. Photographs were scanned, and the band densities corresponding to the amplified native cDNA and the competitor cDNA were calculated as described. After adjusting densitometric measurements for the different size of the two products, the log ratio of the band densities was plotted against the log concentration of competitor added (in attomoles DNA). The x-intercept was interpolated from the calculated trend line to identify the point at which the concentrations of the two products were equal. The quantity of MMP-9 cDNA present in each sample was normalized to β -actin and expressed as a molar ratio for statistical analysis.

Effects of doxycycline on phorbol-stimulated human THP-1 monocytes. Human THP-1 mononu-

clear phagocytes were obtained from the American Type Culture Collection (ATCC; Rockville, Md) and maintained in RPMI-1640 (Gibco BRL) supplemented with 10% fetal calf serum, 10 mmol/L HEPES, and penicillin/streptomycin. Cells were grown in suspension culture at 37°C in a humidified 5% CO₂ atmosphere, then harvested by centrifugation and resuspended at 5×10^5 cells per mL in RPMI-1640 supplemented with 2.5% fetal calf serum and varying concentrations of doxycycline HCl (Sigma Chemical). After 24 hours, cells were harvested by means of centrifugation and resuspended in media containing the same concentration of doxycycline and 100 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma). After an additional 48 hours to allow PMA-induced differentiation, conditioned medium was collected and mixed with 0.1% ethylenediamine tetraacetate (to prevent proMMP auto-activation), then stored at -70°C. Adherent THP-1 cells were lysed in RNA extraction buffer, and total RNA was isolated, as described, before RT-PCR analysis.

Statistical analysis. All blots were processed with a PhotoSmart image scanner (Hewlett-Packard, Palo Alto, Calif) and examined with Phoretix 1D Quantifier version 4.01 densitometry software from NonLinear Dynamics (Newcastle upon Tyne, UK). Data are presented as the mean plus or minus SEM, and all statistical calculations were performed by using SAS for Windows (SAS Institute, Cary, NC), with the level of significance set at 0.05. For comparisons between the doxycycline-treated and the untreated control groups, continuous data were compared by using the Student *t* test, assuming equal variances. Logarithmic transformation was used as necessary to normalize the data distribution. Categorical data were analyzed with the χ^2 test. For the cell culture experiments, correlations between doxycycline concentrations and the measured levels of MMP-9 mRNA (or protein) were calculated by using Pearson correlation coefficients (*r*).

RESULTS

Patient characteristics and doxycycline treatment. No significant differences existed between the two groups of patients in age or aneurysm size (Table II). All eight patients treated with doxycycline were compliant by self-report, and no patient described adverse effects associated with drug treatment. No patient had to decrease the dose of doxycycline or discontinue drug treatment during the 1-week period of study.

Substrate zymography. Gelatin substrate zymography was initially used as a means of detecting the presence of enzymatically active MMP-9 and

Table II. Clinical characteristics of the patient population

<i>Untreated control patients (n = 7)</i>			
<i>Patient number</i>	<i>Sex</i>	<i>Age (years)</i>	<i>AAA (cm)*</i>
1	M	74	4.9
2	M	75	5.9
3	F	76	3.0
4	M	65	9.5
5	M	66	7.0
6	M	85	9.0
7	M	70	8.0
Mean \pm SE	6 M, 1 F	73.6 \pm 2.6	6.76 \pm 0.88
<i>Patients treated with doxycycline (n = 8)</i>			
<i>Patient number</i>	<i>Sex</i>	<i>Age (years)</i>	<i>AAA (cm)</i>
8	M	75	5.0
9	F	69	5.0
10	M	72	5.5
11	M	73	5.0
12	F	79	6.5
13	F	59	4.6
14	M	81	8.0
15	M	67	4.2
Mean \pm SE	5 M, 3 F	72.4 \pm 2.5	5.48 \pm 0.43

*Preoperative measurements of AAA size were obtained by means of computed tomography. There were no significant differences between the untreated control group and the doxycycline-treated group in age or AAA size.

AAA, Abdominal aortic aneurysm; M, male; F, female.

MMP-2 in AAA tissue extracts. As shown by the representative zymogram in Fig 1A, all aneurysm specimens contained gelatinolytic activities corresponding to proMMP-9 (92 kDa), active MMP-9 (88 kDa), proMMP-2 (72 kDa), and active MMP-2 (68 kDa). In four separate experiments, no differences were discernable between patients treated with doxycycline and untreated control patients in the total amount of activity attributable to either MMP-9 or MMP-2. By using inverse densitometry to estimate the fraction of each enzyme present in the active versus latent forms, we detected no difference in the fractional activation of MMP-9. By means of the same analysis, we found that doxycycline treatment was associated with a slight but significant reduction in the activated fraction of MMP-2 (untreated controls, 46.0% \pm 3.7% of total MMP-2 activity; doxycycline-treated, 33.9% \pm 3.6% of total MMP-2 activity; *P* < .05).

Effects of doxycycline on matrix metalloproteinase protein expression in abdominal aortic aneurysm tissue. Given the variables inherent in

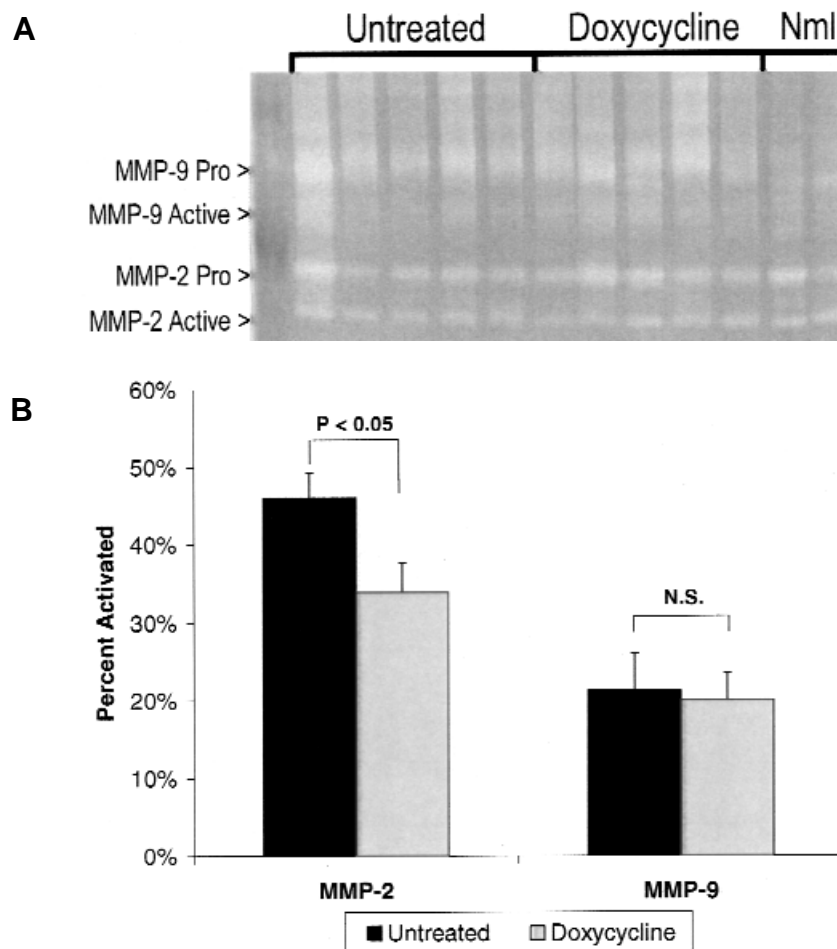


Fig 1. Substrate zymography. Total protein was extracted from the aneurysm wall tissue of patients treated with doxycycline ($n = 5$), untreated control patients ($n = 5$), and two healthy aortas. Equivalent amounts of protein (10 μ g per lane) were electrophoretically separated on a 10% polyacrylamide gel containing 1 mg/mL gelatin. **A**, Gelatinase activity was identified by clear bands on a dark background after staining with Coomassie Blue (representative of four separate experiments). **B**, The mean density of bands corresponding to proMMP-9 (92 kDa), active MMP-9 (88 kDa), proMMP-2 (72 kDa), and active MMP-2 (68 kDa) were measured. There was no difference between groups in total matrix metalloproteinase activity for either MMP-2 or MMP-9 (not shown). The activated fraction of MMP-2 was slightly decreased in samples from patients with abdominal aortic aneurysms treated with doxycycline, compared with those from untreated control patients ($P < .05$), but there was no difference in the activated fraction of MMP-9. NS, not significant.

substrate zymography, immunoblotting of AAA extracts was used as a means of quantifying the effects of doxycycline on aortic wall MMP production. Samples were processed in the presence of proteinase inhibitors to prevent autocatalytic processing during extraction, and the gels were run with an equal amount of total protein in each lane. Immunoblots were performed under denaturing conditions by using a monoclonal antibody to

human MMP-9 and polyclonal antibodies to MMP-2. Immunoreactive MMP-9 was consistently detected in aneurysm tissue from untreated control patients, but not in healthy aortic tissue (Fig 2A). However, aneurysm extracts from patients treated with doxycycline contained appreciably less immunoreactive MMP-9 than those from untreated control patients. When evaluated by means of densitometry (Fig 2B), the level of total extractable

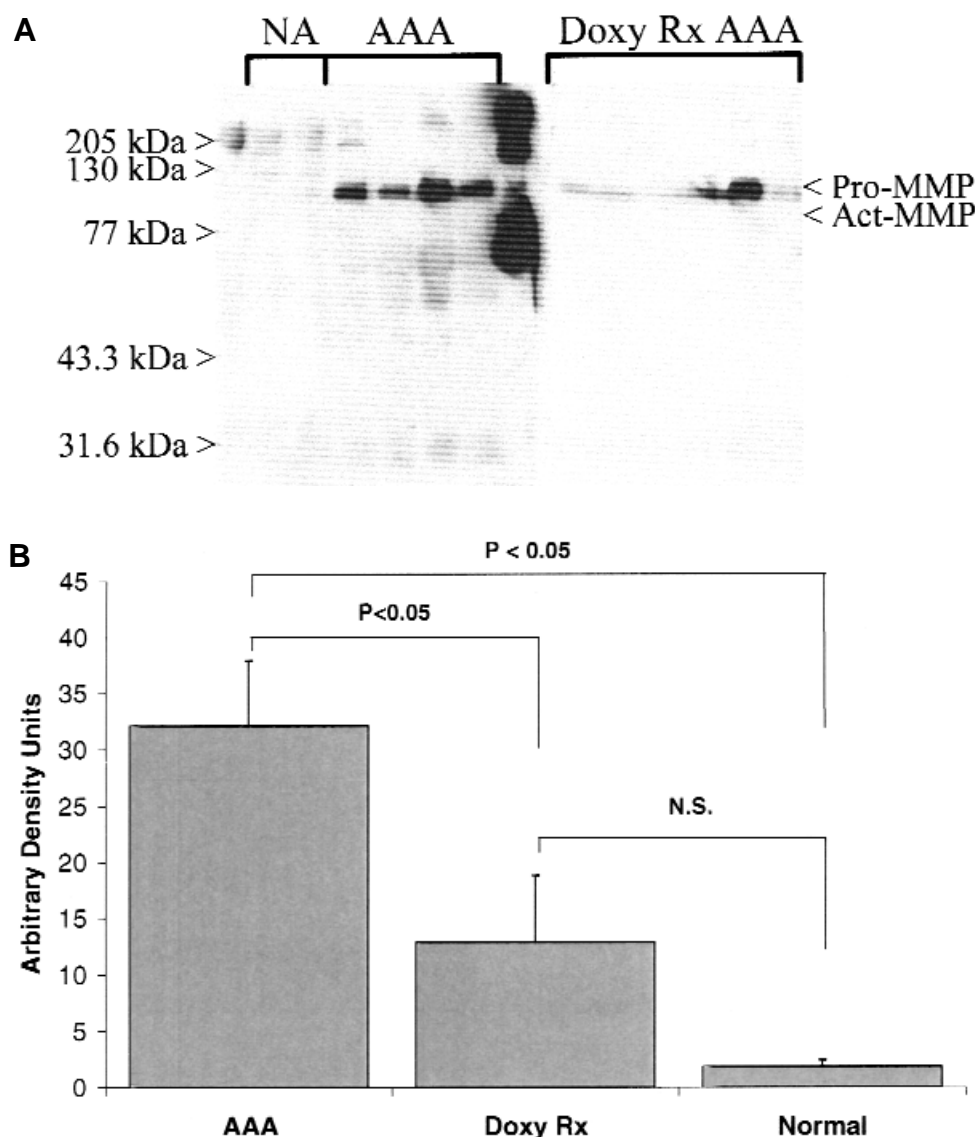


Fig 2. Immunoblotting for MMP-9 in human aortic tissue extracts. Total protein was extracted from the aortic wall tissue of patients treated with doxycycline ($n = 6$), untreated control patients ($n = 4$), and two healthy aortas. Equivalent amounts of protein (100 μ g per lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. **A**, Blots incubated with a monoclonal antibody to human MMP-9 demonstrated a single band migrating at 92 kDa. **B**, By using band densitometry, samples from untreated patients with abdominal aortic aneurysms contained significantly more MMP-9 protein than those from healthy aortas ($P < .05$). Samples from patients with an abdominal aortic aneurysm treated with doxycycline exhibited a significant decrease in the amount of detectable MMP-9 protein, compared with those from untreated patients with an abdominal aortic aneurysm ($P < .05$). There was no significant difference between the amount of MMP-9 in samples from the doxycycline-treated group and those from normal aortas. *NS*, not significant.

MMP-9, as measured in arbitrary density units (ADU), was significantly higher in the untreated aneurysm control group than in both aortas of patients treated with doxycycline ($P < .05$) and

healthy aortas ($P < 0.02$; untreated AAAs, 32.1 ± 5.85 ADU; doxycycline-treated AAAs, 12.9 ± 5.93 ADU; healthy aortas, 1.87 ± 0.59 ADU). Indeed, the difference in MMP-9 between aortas of patients

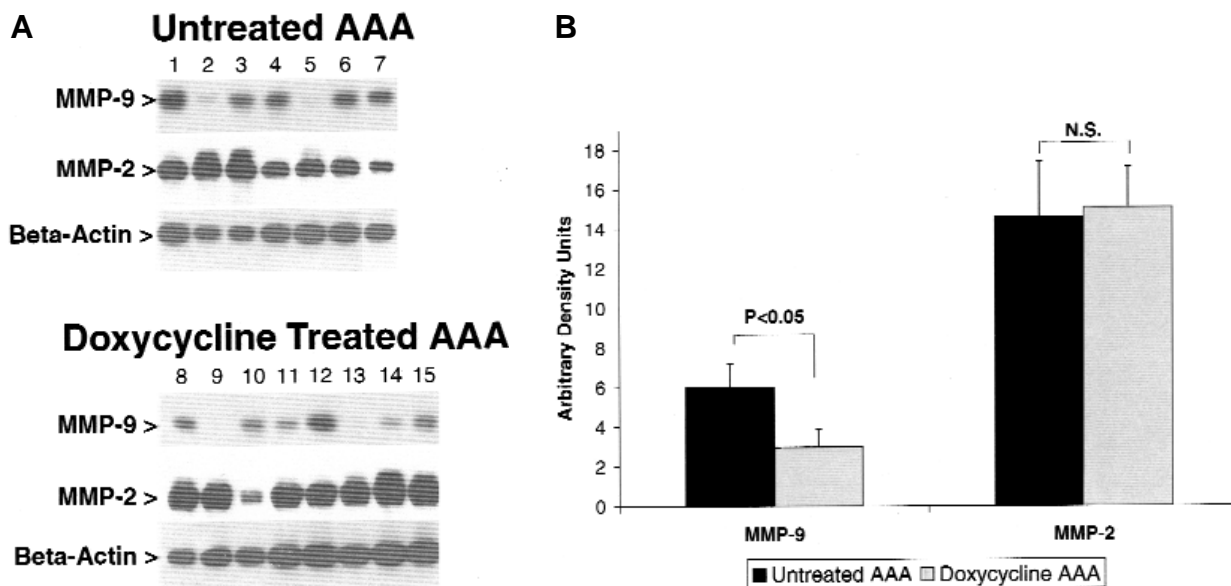


Fig 3. Reverse transcription-polymerase chain reaction and Southern blot analysis of MMP-9 and MMP-2 RNA in human aneurysm tissue. Total RNA was extracted from aneurysm wall tissue of patients treated with doxycycline and untreated control patients, then amplified by reverse transcription-polymerase chain reaction using primer pairs specific for MMP-9, MMP-2, and β -actin. **A**, Reverse transcription-polymerase chain reaction products were confirmed by means of Southern blot with specific probes within the amplified sequence. Although there was some variation in MMP-9 and MMP-2 production between specimens, there was a recognizable decrease in the expression of MMP-9 between samples from patients treated with doxycycline and untreated control patients. **B**, Densitometric analysis of MMP-9 and MMP-2 relative to β -actin. There was a significant decrease in the amount of MMP-9 messenger RNA in samples from the doxycycline-treated group, compared with untreated controls ($P < .05$). The difference between groups for MMP-2 messenger RNA was not significant. NS, not significant.

treated with doxycycline and healthy aortas was not statistically significant. Also, the one specimen in the doxycycline-treated group containing a substantial amount of MMP-9 was obtained from the largest aneurysm in the series (8.0 cm in diameter). In contrast to the results obtained for MMP-9, no significant difference in the amount of this enzyme extracted from AAA tissues in the doxycycline-treated group and the untreated control group was revealed by means of immunoblot analysis of MMP-2 (data not shown).

Treatment with doxycycline is associated with a reduction in MMP-9 messenger RNA. The observed reduction in extractable MMP-9 protein in patients treated with doxycycline was somewhat surprising, because of the abundant expression of this proteinase in AAA tissue and the relatively short period of doxycycline treatment. To clarify whether this effect was caused by a concomitant reduction in MMP-9 gene expression, we used RT-PCR to exam-

ine the effects of treatment with doxycycline on aortic wall expression of MMP-9 and MMP-2.

With Southern hybridization of cDNA products amplified from AAA tissue-derived RNA, bands corresponding to both MMP-9 and MMP-2 mRNA were identified in all samples (Fig 3A). After densitometric measurements and normalizing the samples to β -actin mRNA as a constitutively expressed control, a significant reduction in aneurysm tissue expression of MMP-9 between patients treated with doxycycline and untreated control patients, but no difference in MMP-2, was revealed by means of semi-quantitative analysis (Fig 3B). Thus, the mean β -actin normalized value of MMP-9 in aneurysm tissue from the eight patients treated with doxycycline was 2.98 ± 0.90 arbitrary relative density units (ARDU), a value less than half of that observed in aneurysm tissues from untreated control patients (6.02 ± 1.18 ARDU; $P < .05$, Student *t* test). In examining data from individual patients, we noted that five of seven

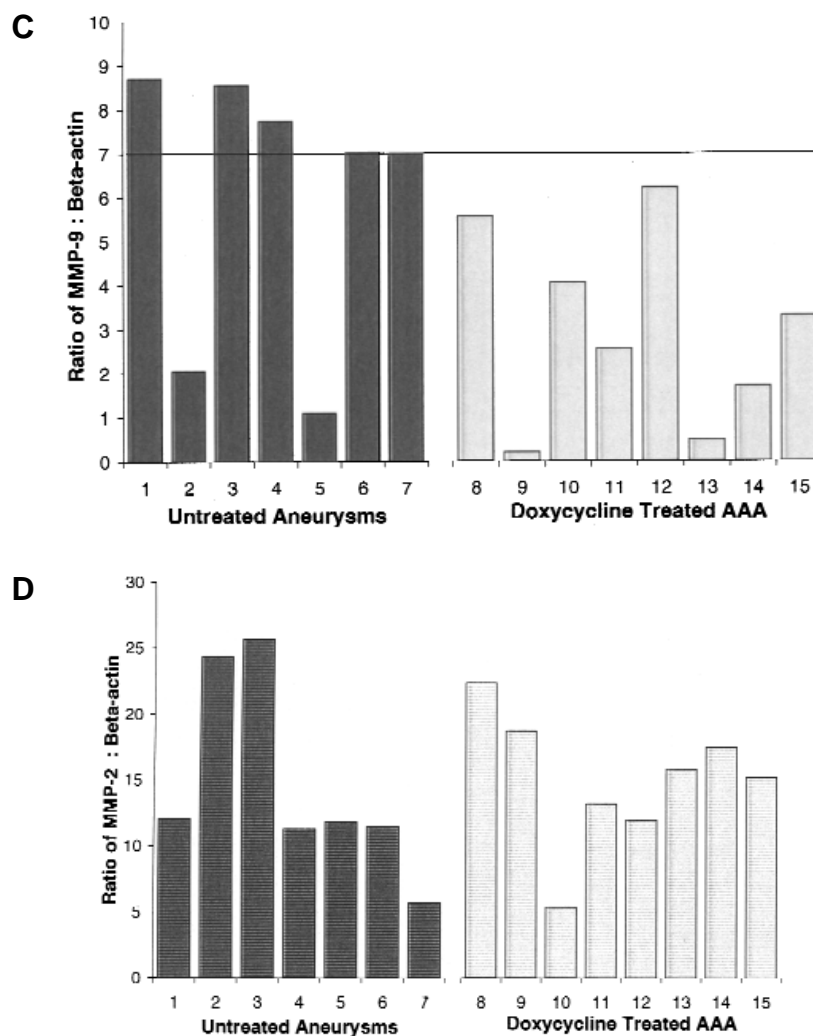


Fig 3. Cont'd. C, Densitometric measurements of MMP-9 messenger RNA for individual specimens shown in A. Five of the seven specimens from untreated patients with abdominal aortic aneurysms had normalized densities greater than or equal to 7 (horizontal line), whereas none of the eight specimens from patients treated with doxycycline had normalized densities greater than this value ($P < .01$; χ^2 analysis). D, Densitometric measurements of MMP-2 messenger RNA for the same samples shown in C. There was no detectable trend differentiating the two groups.

specimens from those patients in the untreated control group had β -actin normalized MMP-9 values of 7 ARDU or more, whereas none of the eight specimens from patients treated with doxycycline exhibited a relative density greater than 6.25 ARDU (Fig 4A). With the empirically-defined threshold of 7 ARDU to compare individual samples, a significantly greater number of patients treated with doxycycline had low levels of MMP-9 expression, compared with untreated control patients ($P < .01$ by means of χ^2 analysis). On the other hand, there were no apparent differences in the relative levels of MMP-2 expression

between individual patients in the doxycycline-treated and untreated control groups (Fig 4B).

To quantify the results of RT-PCR analysis for MMP-9 with more accuracy, we developed competitor cDNA constructs for both human MMP-9 and β -actin.^{19,25,54,55} By using the same primer pairs for MMP-9, competitive PCR reactions resulted in two amplification products readily distinguishable by size on agarose gels (competitor cDNA, 347 bp; native cDNA, 243 bp). Reactions performed in the presence of increasing concentrations of the competitor resulted in a progressively diminished amount of the

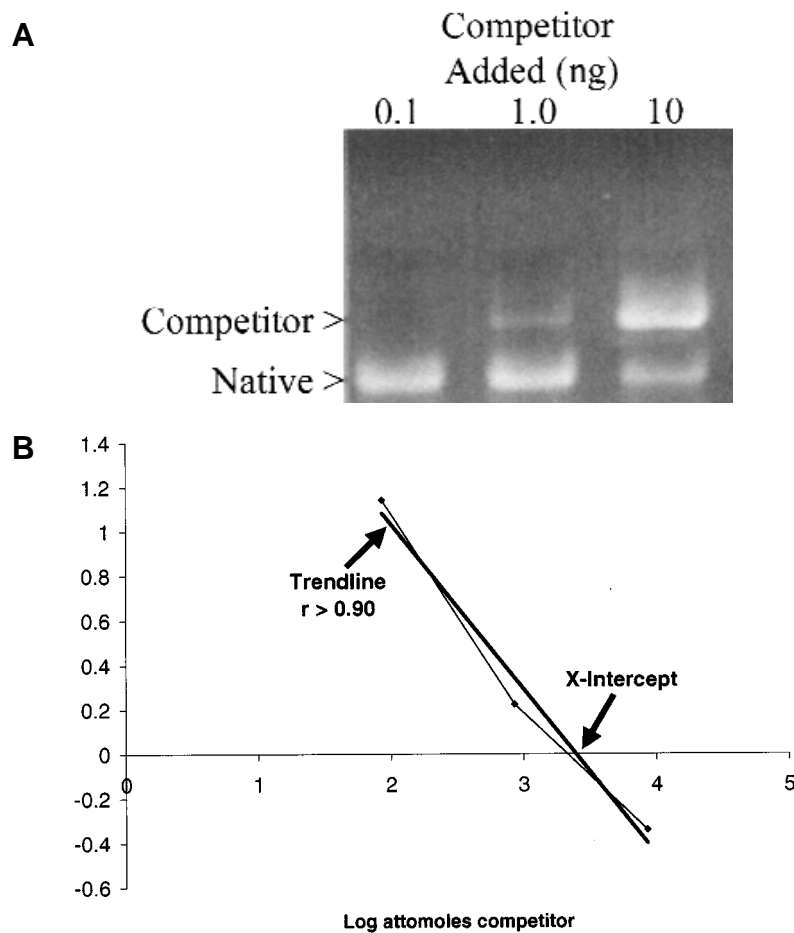


Fig 4. Representative results of competitive reverse transcription-polymerase chain reaction analysis for MMP-9. Native complementary DNA prepared by reverse transcription from AAA tissue was amplified in a series of reactions containing increasing amounts of an MMP-9 competitor complementary DNA, which competes with the native complementary DNA for a fixed quantity of oligonucleotide primers. **A**, By design, the two products of polymerase chain reaction amplification (at least 100 base pair different) can be distinguished by size after agarose electrophoresis. **B**, The amount of competitor added (in moles) was plotted against the density ratio of the two bands by using a log/log scale. After linear regression to ensure a correlation coefficient less than -0.90 , the x-intercept of the plotted data was determined to be the point at which the (known) amount of competitor and the (unknown) amount of native complementary DNA are equivalent. For accurate comparisons, the amount of MMP-9 complementary DNA in each sample was normalized to the amount of β -actin measured in a parallel series of competitive reactions, and expressed as an mol/mol ratio.

native cDNA product (Fig 5A). Densitometric data were then used as a means of constructing a plot of the known competitor concentration to the ratio of the two cDNA products on a log-log scale, and linear regression was performed as a means of identifying the point of equivalence at the x-intercept (ie, corresponding to the amount of native cDNA present in the original sample; Fig 5B). The amount of MMP-9 mRNA present in each sample was then

normalized to the amount of β -actin measured in a parallel series of competitive reactions.

Fig 6 summarizes the results of competitive RT-PCR analysis for MMP-9 (expressed in mol MMP-9/mol β -actin) in AAA specimens from five patients treated with doxycycline and four untreated control patients. The mean concentration of MMP-9 mRNA in the doxycycline-treated group was 1.3 ± 0.5 mol/mol β -actin, compared with 7.2 ± 3.1 mol/mol

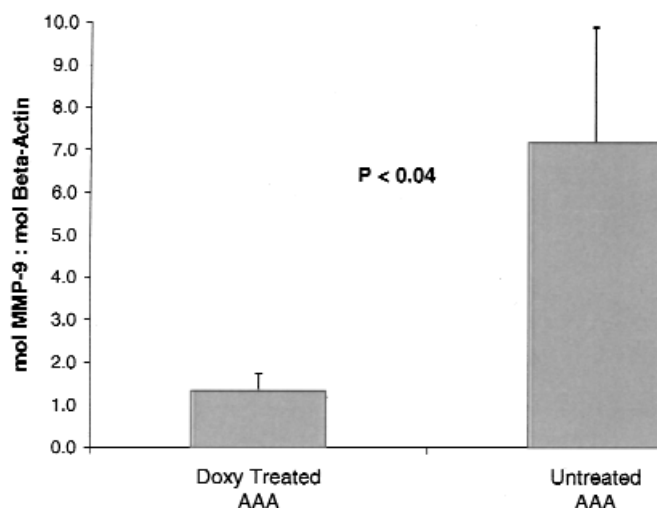


Fig 5. Summary of competitive reverse transcription-polymerase chain reaction analysis for MMP-9 in abdominal aortic aneurysm tissue. MMP-9 messenger RNA in aneurysm tissue was quantified by means of competitive reverse transcription-polymerase chain reaction as shown in Fig 4, by using total RNA obtained from five patients treated with doxycycline and four untreated control patients. Data were expressed as a molar ratio of MMP-9 to β -actin. Treatment with doxycycline was associated with a 5.5-fold (81.9%) reduction in the expression of MMP-9, compared with the untreated control group ($P < .04$; Student t test).

β -actin in the untreated control group ($P < 0.04$, Student t test). Thus, treatment with doxycycline was associated with a 5.5-fold (81.9%) reduction in the relative expression of MMP-9 in aneurysm tissue, even after only 1 week of therapy.

Doxycycline suppresses PMA-stimulated MMP-9 expression in cultured human THP-1 mononuclear phagocytes. Because treatment with doxycycline was associated with a reduction in aortic wall expression of MMP-9 protein and mRNA, and because mononuclear phagocytes are the principal source of MMP-9 in human AAA tissue,¹⁶ we used a human monocytic cell line to further examine the regulation of MMP-9 expression by doxycycline in vitro. THP-1 cells were preconditioned with doxycycline for 24 hours at 0, 2.5, 5, 10, and 12 $\mu\text{mol/L}$, a range of concentrations corresponding to tissue drug levels measured during therapy.^{46,56,57} In the continuing presence of doxycycline, the cells were then exposed to 100 nmol/L PMA, an agent known to stimulate MMP-9 production in mononuclear phagocytes.⁵⁸⁻⁶⁰ At all concentrations of doxycycline tested, THP-1 cells exhibited a normal morphologic appearance after PMA stimulation, with differentiation to an adherent phenotype and cell spreading on the substratum (data not shown). Suppression of PMA-stimulated MMP-9 mRNA expression (normalized to β -actin) was demonstrated by means of

RT-PCR analysis at doxycycline concentrations as low as 5 $\mu\text{mol/L}$ (Fig 6A). This effect was significant and dose-dependent, with a Pearson correlation coefficient of $r = -0.88$ ($P < .05$). Conditioned media from these experiments were also examined by means of Western blot to detect MMP-9 protein. As in previous studies, four immunoreactive bands were detected, which correspond to the proenzyme and active forms of MMP-9, both free and complexed to TIMP-1 (data not shown).¹⁶ By using the sum of all four bands to calculate the total amount of MMP-9 present, Fig 6B shows that there was also a concentration-dependent decrease in the overall amount of MMP-9 produced by cells exposed to doxycycline ($r = -0.90$; $P < .04$). A similar effect of doxycycline on the amount of enzymatically active (88 kDa) MMP-9 present in the conditioned medium ($r = -0.95$; $P < .02$) was revealed by means of gelatin zymography (Fig 6C).

DISCUSSION

The present investigations were undertaken with the view that doxycycline may eventually be useful as an MMP inhibitor in the pharmacologic treatment of patients with a small asymptomatic AAA. This notion follows previous studies emphasizing the critical role of connective tissue degradation in aneurysmal degeneration, the demonstration that human

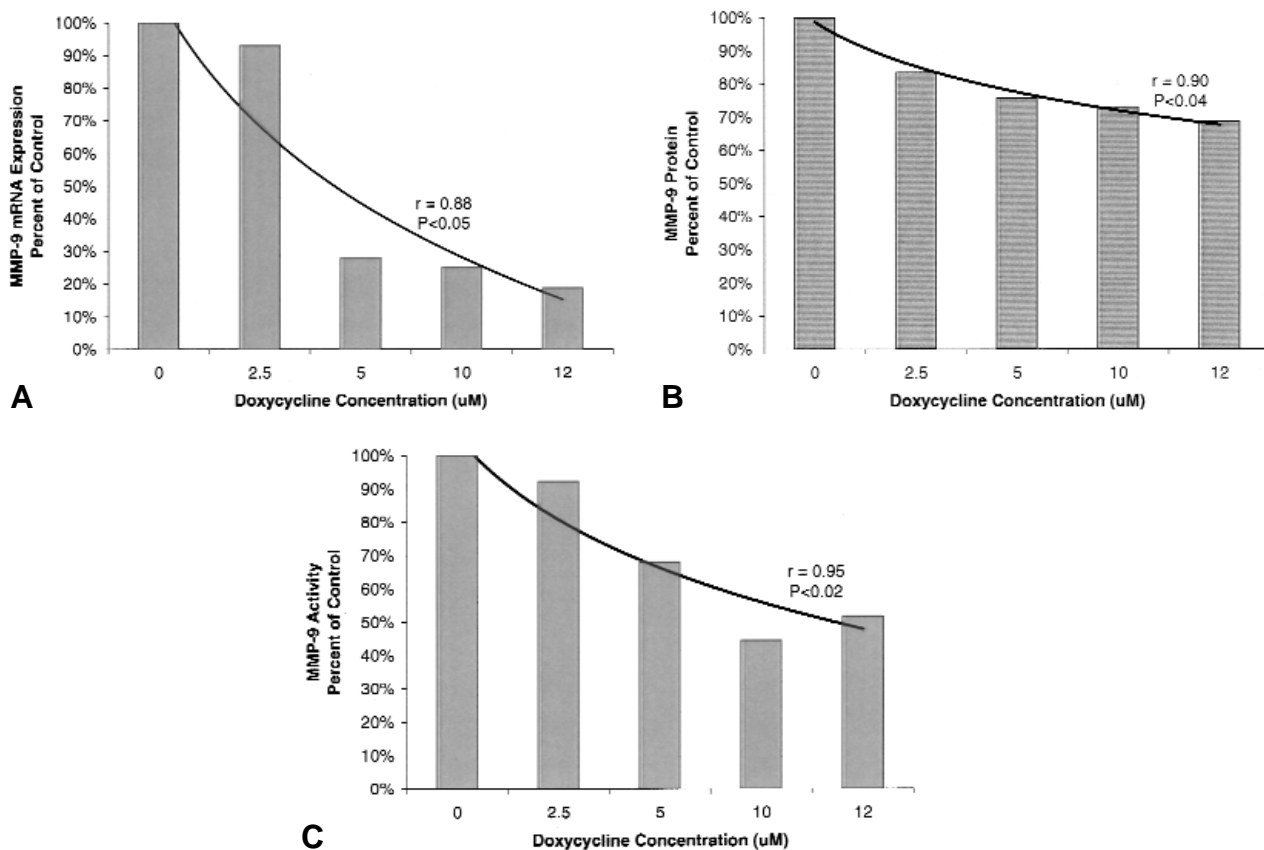


Fig 6. Effect of doxycycline on MMP-9 production in phorbol 12-myristate 13-acetate (PMA)-stimulated THP-1 cells. An equivalent number of human THP-1 mononuclear phagocytes were preincubated with either 0, 2.5, 5, 10, or 12 $\mu\text{mol/L}$ doxycycline for 24 hours, then stimulated for 48 hours with 100 nmol/L PMA in the continuing presence of doxycycline. At all concentrations of doxycycline tested, cells exhibited a normal morphologic appearance following PMA stimulation with differentiation to an adherent phenotype (not shown). **A**, Cellular expression of MMP-9 and β -actin messenger RNA was measured by means of reverse transcription-polymerase chain reaction, with data normalized to β -actin and expressed as a percentage of control (cells not exposed to doxycycline). Doxycycline induced a concentration-dependent inhibition of MMP-9 messenger RNA expression ($r = -0.88$; $P < .05$). **B**, Conditioned media were normalized to total protein concentration and examined by means of Western blot with monoclonal antibodies to human MMP-9. Total immunoreactive MMP-9 was measured by means of densitometry and expressed as a percentage of control. Doxycycline induced a concentration-dependent decrease in the amount of MMP-9 protein secreted into the conditioned medium ($r = -0.90$; $P < .04$). **C**, Equal amounts of conditioned media were examined by means of gelatin zymography. Total gelatinolytic activity attributable to MMP-9 was measured by means of inverse densitometry and expressed as a percentage of control. Doxycycline (not present in the substrate buffer during zymography) induced a concentration-dependent decrease in MMP-9 activity secreted into the conditioned medium ($r = -0.95$; $P < .02$).

and experimental AAAs are associated with chronic inflammation and increased local production of MMPs, and the recognition that tetracycline derivatives have efficacy as MMP inhibitors, both in vitro and in vivo. It has also been demonstrated that doxycycline has beneficial effects in experimental

models of AAA, acting to suppress aortic wall elastin degradation, MMP activity, and aneurysmal dilatation.^{34-36,61} The background, rational, and current strategies being used to develop pharmacological approaches for patients with a small asymptomatic AAA have recently been reviewed.^{1-4,61-63}

The results presented here provide important new information from a number of different perspectives. First, we provide experimental evidence that treatment with doxycycline can influence aortic wall expression and activity of MMPs in patients with an AAA. Previous studies have suggested that tetracyclines can localize to atherosclerotic plaques^{64,65} and that tetracycline rapidly penetrates into the aneurysmal aorta after intravenous injection,⁶⁶ but it has not been previously demonstrated that doxycycline can effectively inhibit MMPs in the complex tissue environment of human AAAs. Our observations also indicate that these effects can be achieved by means of relatively short-term treatment with doxycycline at clinically accepted doses, analogous to those used in long-term antimicrobial therapy and other conditions in which doxycycline has been used as an MMP inhibitor (eg, periodontitis and rheumatoid arthritis).⁴⁶ Doxycycline was well tolerated in our patient population, and no patient had adverse effects that required discontinuation of the medication. This provides a useful guideline for further studies of doxycycline in patients with an AAA, both to determine whether similar effects on MMP production can be achieved with lower dose schedules (eg, 20 mg orally twice a day),^{40,47,67} or eventually, whether sustained treatment has an influence on the expansion of small asymptomatic AAAs.⁶³

Second, this study shows that, in addition to its recognized effects as a direct MMP antagonist, the mechanisms of action by which doxycycline might suppress connective tissue degradation include substantial effects on MMP gene expression (ie, MMP-9) and extracellular activation (ie, MMP-2). Through this complementary combination of mechanisms, treatment with doxycycline would be expected to be particularly effective in influencing aortic wall MMP activities *in vivo*. Doxycycline and other tetracyclines are relatively nonselective in their direct inhibition of various MMPs, whereas their concomitant effects on MMP production and activation appear to be potentially more selective. In this study, we examined two metalloproteinases with elastolytic activity, MMP-9 and MMP-2, both of which are implicated in the pathophysiology of aneurysmal degeneration.¹⁰ Selective inhibition of MMP-9 gene expression in mononuclear phagocytes may be particularly advantageous, because production of this enzyme is elevated 10-fold in human AAA tissue.^{16,18-20,25,26} Although this may be another advantage of doxycycline over more specific MMP antagonists, further studies are underway to elucidate whether doxycycline influences the expression of other MMPs involved in progressive aneurysmal degeneration, such as interstitial

collagenases (MMP-1, MMP-8, and MMP-13), stromelysin (MMP-3), and macrophage metalloelastase (MMP-12).

In contrast to doxycycline's effects on MMP-9, we did not observe an effect of doxycycline on aortic wall expression of MMP-2. A slight reduction in the activated fraction of MMP-2 in extracts of AAA tissue from patients treated with doxycycline was revealed by means of semiquantitative analysis of substrate zymograms; this may be at least partially explained by proMMP-2 undergoing a unique pattern of extracellular activation dependent on cell surface binding, interactions with TIMP-2, and processing by membrane-type metalloproteinases (MT-MMPs).⁶⁸⁻⁷⁰ As nonselective antagonists of MMP activity, doxycycline and other tetracyclines might be expected to inhibit proMMP-2 activation by suppressing MT-MMP activity. Inhibition of pericellular proteolysis through this mechanism may be an important pathway for the capacity of tetracycline derivatives to suppress matrix degradation in a variety of clinical circumstances. In contrast, the activation of MMP-9 is thought to involve more diverse pathways, involving other MMPs, oxidative processes, and serine proteases such as plasmin and urokinase-type plasminogen activator.

A third observation from this study is that doxycycline reduces the expression of MMP-9 in phorbol-stimulated human THP-1 mononuclear phagocytes in culture. The possibility that tetracyclines can regulate MMP expression is supported by previous studies in cultured cells, as demonstrated for MMP-2 in skin keratinocytes,⁴⁹ MMP-8 in rheumatoid synovial fibroblasts and endothelial cells,⁵⁰ and MMP-9 in human umbilical vein endothelial cells.⁵¹ In pathologic chondrocytes isolated from osteoarthritic joints, Shlopov et al have also demonstrated that doxycycline induces a downregulation in steady-state mRNA for all three mammalian collagenases (ie, MMP-1, MMP-8, and MMP-13).⁵² Although we have observed indirect evidence for a decrease in macrophage MMP-9 production after treatment with doxycycline in the elastase-induced rat model of AAA,³⁴ the *in vitro* experiments described here provide the first direct evidence for an inhibitory effect of tetracyclines on MMP-9 expression in mononuclear phagocytes. Monocyte-derived tissue macrophages are the principal source of MMP-9 in human and experimental aneurysm tissues and other disorders characterized by chronic inflammation and connective tissue destruction. Also, stimulation by phorbol ester reflects one of the dominant molecular pathways acting to induce MMP-9 transcription in monocytic cells (ie, activation of protein kinase C and

increased promoter activity regulated by activator protein-1 complexes).⁷¹ Thus, the demonstration that doxycycline suppresses phorbol-stimulated expression of MMP-9 in THP-1 cells, particularly at concentrations typically achieved in tissue during systemic antibiotic treatment (4 to 20 $\mu\text{mol/L}$),^{46,56,57} supports the likelihood that this is one of the mechanisms by which doxycycline regulates MMP-9 expression in human AAAs in vivo. Experiments in progress will help determine whether the molecular mechanisms of this effect reside in a suppression of MMP-9 transcription or mRNA stability, whether this mode of regulation involves alterations in specific signal transduction pathways or transcriptional elements, and how doxycycline influences other metalloproteinases and inhibitors normally expressed by mononuclear phagocytes.

There were a number of limitations inherent to this study. For example, the study design was necessarily correlative in nature, because we were only able to evaluate the effects of systemic doxycycline treatment by comparing measures of MMP production in tissues obtained from two concurrent groups of patients. Although these patients were not formally randomized to treatment with doxycycline or a placebo, the patients in each group were studied during the same period, and they were not selected by any specific criteria other than the contingencies of preoperative scheduling. The two patient groups were also found to be comparable in age and mean aneurysm size. McMillan et al reported a correlation between aortic wall MMP-9 mRNA expression and aneurysm size, at least in small and medium AAAs (5.0 to 6.9 cm in diameter).²⁵ They also found that MMP-9 expression is decreased in large aortic aneurysms (larger than 7.0 cm in diameter).²⁵ In examining the individual patients in our study, we found four AAAs larger than 7.0 cm in diameter in the untreated control group, but only one in the doxycycline-treated group. By using the correlations drawn by McMillan et al, if there were any bias introduced by discrepancies in the size of individual aneurysms, we would have expected it to cause a lower level of MMP-9 expression in the untreated control group. Our results pointed to an effect in the opposite direction, substantiating the conclusion that treatment with doxycycline reduces AAA wall expression of MMP-9. Because our results may still have been biased by unmeasured differences between the two groups in other clinical characteristics, further studies are recommended to confirm these findings.

A second limitation in design was that this study involved only short-term treatment with doxycycline

in a relatively small number of patients. Although this was imposed by practical concerns in treating patients scheduled for elective AAA repair, it would be interesting to evaluate how more sustained treatment with doxycycline might affect aortic wall MMP production in a larger series. This may not be feasible outside a prospective controlled trial in patients with a small AAA, but one of the promising directions for future investigation is suggested by recent evidence that circulating levels of MMP-9 are increased in patients with an AAA.²⁶ Thus, it will be interesting to determine whether prolonged treatment with doxycycline can influence serial measures of plasma MMP-9, as a surrogate marker of enzyme production within the aneurysm wall itself.

Finally, two technical limitations of this study were related to the difficulty in measuring MMP enzymatic activities in protein extracts from human AAA tissue by means of *in vitro* assays and the apparent discrepancy between results obtained by means of substrate zymography and other techniques. Although *in vitro* activity assays would have been useful in demonstrating whether doxycycline treatment has a direct influence on tissue MMP activity, we have found that accurate measures of MMP activity in protein extracts from AAA tissue are complicated by the co-extraction of excess TIMP-1, a naturally occurring MMP inhibitor that is overexpressed in human AAAs (Liao and Thompson, unpublished observations).^{12,16,18,19} A second concern was raised by the apparent lack of effect of doxycycline treatment as determined by means of gelatin zymography. Although zymography is usually a sensitive technique for the detection of proteinase activities, it is often quite insensitive as a measure of the activity present in different samples. We found that resolution of gelatinase activities in AAA extracts required a 10-fold reduction in the amount of total protein loaded per lane, as compared with immunoblots, and that inverse densitometry was an insufficient means of measuring the 2.5-fold difference in MMP-9 that we observed with immunoblot analysis. We believe the results obtained by means of immunoblotting are a more accurate reflection of the events that occur in the aortic wall tissue of patients treated with doxycycline, because this approach is subject to fewer variables than zymography and because the quantitative results of immunoblotting were in accord with those obtained by means of RNA analysis. In contrast to the use of densitometric analysis as a means of estimating total MMP activity, this approach was more accurate in comparing the fractional activation of each enzyme because it was based on an internal control calculated for each enzyme in each sample.

A consistent reduction in the activated fraction of MMP-2 after doxycycline treatment, but no effect on the activation of MMP-9, was revealed by means of analysis of data obtained in four different experiments. Although the difference in fractional activation of MMP-2 was statistically significant between groups, the overall effect was still only a slight reduction, compared with that of untreated control patients. Because active MMP-2 binds with high avidity to the extracellular matrix in AAA tissue, a greater difference might have been obtained with harsher tissue extraction conditions.⁷⁰ Further studies will, therefore, be needed to clarify how doxycycline influences aortic wall MMP activities detected by means of substrate zymography.

In summary, the present study establishes that treatment with doxycycline is associated with significant inhibitory effects on the local expression and post-translational processing (activation) of two MMPs considered to be important in the pathogenesis and progression of human aortic aneurysms. With the findings of previous studies, these findings indicate that treatment with doxycycline may be a useful approach to begin prospective clinical trials aimed at reducing aneurysm expansion in patients with small asymptomatic AAAs.

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DISCUSSION

Dr William H. Pearce (Chicago, Ill). Dr Curci should be commended for his excellent presentation. Before I make my comments, I would like to point out that Drs Thompson and Reilly are recipients of the Lifeline Foundation Award, and it is clear that their basic science research has been brought to human trials at this point.

This paper is an excellent example of translational research. A number of investigators, beginning with Busuttil, have made observations that collagenolytic activity is present in aortic aneurysm walls. Today, we know that those enzymes are members of the metalloproteinase families and, in particular, MMP-2 and MMP-9. Dr Thompson, Dr Sicard and his colleagues, and Dr Reilly have pursued this in an animal model and found that doxycycline inhibited aneurysm growth. It was only logical then that they move from the bench to the bedside and try this in humans. And, in fact, they used 100 mg twice a day and demonstrated marked reductions in enzyme levels in the arterial wall. What is interesting to me is that the large aneurysms formed their control group. And from studies in our own laboratory by Bill McMillan, these large aneurysm groups have been found to have the lowest levels to begin with, so their findings are even more dramatic.

The studies that they have performed are well-designed, and the techniques are impeccable. I have several questions for the authors, only as a matter of discussion.

First, in addition to the two matrix metalloproteinases studied, MMP-2 and MMP-9, have you studied others? I note that you have recent publications about MMP-12 and MMP-13. This is a very large family, and presumably doxycycline will affect many of these enzymes.

Second, have you studied any patients in a longitudinal fashion? To prove the hypothesis, the reduction of aneurysm growth or a marker such as serum MMP-9 should decline.

Finally, we know from studies in patients with rheumatoid arthritis that doxycycline or one of the modified doxycyclines has been very effective in the treatment of rheumatoid arthritis. Do you anticipate any problems with long-term doxycycline treatment when we begin clinical trials in patients with small aneurysms?

I believe this paper is a model for translational research, and I compliment the St. Louis group on their persistence in bringing this to us today. And I'd like to thank the Society for the privilege of discussing this paper. Thank you.

Dr Robert W. Thompson. Thank you very much, Dr Pearce.

The first question you asked was about whether we have studied other matrix metalloproteinases. Today, we've only looked at MMP-2 and MMP-9 in detail. We do have a great interest in MMP-12, macrophage elastase, which is also markedly elevated in aneurysm tissue, but we have not yet done detailed studies in human tissue on the effect of doxycycline treatment on MMP-12. We will continue to extend this to other matrix metalloproteinases in the family that may be involved in aneurysm disease in the next year.

To answer your second question regarding longitudinal follow-up, that really is the prelude to a longer-term clinical trial. We have shown now that we can, in even 1 week of treatment, affect matrix metalloproteinase activity and production in human aneurysm tissue. This is an important first step, but it is, in a sense, preliminary data. What we really

need to do now is show that by affecting matrix metalloproteinase activity in human aneurysm tissue we can actually influence the natural history of a small aortic aneurysm. This will require a large randomized clinical trial. We are currently in the process of organizing and beginning such a trial. Dr Timothy Baxter at the University of Nebraska and myself, with physicians from several other centers in the Midwest, have started a pilot phase study, and we hope to be entering a larger-scale study within approximately 6 to 9 months. This will naturally take approximately 4 or 5 years of follow-up, I think, before we'll have an answer. But this is the critical question, and we don't know yet if any form of pharmacotherapy will affect the natural history of small aortic aneurysms.

Doxycycline and even low-dose doxycycline in another chemical formulation have been used as matrix metalloproteinase inhibitors in clinical trials for other conditions, such as rheumatoid arthritis. Osteoarthritis of the knee is also being studied in a large-scale clinical trial with doxycycline. And periodontal disease, in which the use of tetracyclines really began as matrix metalloproteinase inhibitors, has also been studied for the last 13 years. In these situations, doxycycline has been used successfully as an matrix metalloproteinase inhibitor, and there have not been major problems with adverse effects of the antibiotic. There are some drug-specific adverse effects, such as photosensitivity, and there are some incidents of gastrointestinal disturbance with doxycycline, but these have been relatively minor. Particularly in the trials of rheumatoid arthritis and osteoarthritis, the patient populations are in many ways similar to the group of patients we would be proposing to treat with aneurysms, so I think that this is a promising route by which we can begin to test this hypothesis.

Thanks once again.

Dr David R. Jackson (Northhampton, Mass). This is a very intriguing and interesting paper. Many of us in this audience treat chronic, nonhealing diabetic and nondiabetic foot ulcers. Recent wound healing research has shown that the metalloproteinase MMP-9 is highly prevalent in many of these chronic wounds. Some of the researchers have indicated that they think that the effect on the collagenases in the wound to decrease the effect on the collagen matrix formation may be part of the reason why these chronic wounds don't heal. I wonder if the authors have any information about whether doxycycline has been used in the treatment of chronic foot ulcers?

Dr Alexander W. Clowes (Seattle, Wash). I want to commend the authors on a very interesting study and underscore an important new development, a set of surro-

gate endpoints to test out their pharmacology first before going for the final long-range endpoint. I think this is a very important principle.

I would like to ask about carotid disease, another disease that involves disruption of the wall, but is limited to the intima. One would suppose that giving some form of tetracycline/doxycycline might suppress the disruption of the fibrous cap, and one would have the opportunity also to determine whether the drug could affect the proteases that are involved in this process. Have you had a look at carotid endarterectomy specimens to see whether you can see the same kind of effect of doxycycline on the proteases in those lesions?

Dr Joseph M. Van De Water (Macon, Ga). Is there an application of this research to other conditions in which you have degradation of elastin, such as bronchiectasis or maybe even the common hernia?

Dr Thompson. Thank you for your questions.

Dr Jackson asked about wound healing and the use of doxycycline as an matrix metalloproteinase inhibitor. Matrix metalloproteinases, not only MMP-2 and MMP-9 but many others, particularly collagenases, are clearly involved in wound healing. Doxycycline and other tetracyclines are broad spectrum matrix metalloproteinase inhibitors. By means of the mechanism by which they are direct antagonists, they can affect all members of the matrix metalloproteinase class. We don't have any specific data on wound healing, but these enzymes are being studied in great detail by others.

Dr Clowes asked about the carotid plaque, and I think that this is a very intriguing problem that parallels aneurysm disease. Metalloproteinases are clearly involved in the breakdown of matrix in the fibrous cap of atherosclerotic plaques, wherever they are, and can lead to plaque vulnerability, rupture, and clinical complications. Clearly, an matrix metalloproteinase inhibitor may be a promising clinical therapeutic approach for this problem. We have done no studies on carotid lesions in particular; but a parallel study, of the nature we presented today, would be very intriguing and promising.

Finally, we have done no studies on hernia or other specific conditions related to elastin, but any of the matrix metalloproteinases that break down elastin—there are four in the family that do so—can be inhibited by tetracycline or other members of this class. So, although we have no data, this would be another promising area for future research.

I thank the Society, once again, for the opportunity to present.